Topical Calcipotriol Plus Imiquimod Immunotherapy for Nonkeratinocyte Skin Cancers

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Nonkeratinocyte cutaneous malignancies, including breast cancer cutaneous metastasis and melanoma in situ, are often poor surgical candidates. Imiquimod (IMQ), a toll-like receptor 7 agonist that activates innate immunity in the skin, is used to treat these cutaneous malignancies. However, IMQ's modest effect on the activation of adaptive immunity limits its efficacy as a monotherapy. In this study, we demonstrate that topical TSLP cytokine inducers—calcipotriol and retinoic acid—synergize with IMQ to activate CD4$^+$ T-cell immunity against nonkeratinocyte cutaneous malignancies. Topical calcipotriol plus IMQ treatment reduced breast tumor growth compared with calcipotriol or IMQ alone ($P < 0.0001$). Calcipotriol plus IMQ—mediated tumor suppression was associated with significant infiltration of CD4$^+$ effector T cells in the tumor microenvironment. Notably, topical calcipotriol plus IMQ immunotherapy enabled immune checkpoint blockade therapy to effectively control immunologically cold breast tumors, which was associated with induction of CD4$^+$ T-cell immunity. Topical treatment with calcipotriol plus IMQ and retinoic acid plus IMQ also blocked subcutaneous melanoma growth. These findings highlight the synergistic effect of topical TSLP induction in combination with innate immune cell activation as an effective immunotherapy for malignancies affecting the skin.

INTRODUCTION

Several nonkeratinocyte cutaneous malignancies are considered poor candidates for surgical excision owing to the location of the cancer, the extent of the disease, and the morbidities associated with their surgery. These cutaneous malignancies include breast cancer cutaneous metastasis and cutaneous melanoma, especially melanoma in situ lesions and lentigo maligna (Bichakjian et al., 2011; Lookingbill et al., 1993). Breast cancer cutaneous metastasis or chest wall disease of breast cancer (CWD) mainly presents as subcutaneous nodules on the anterior chest wall (De Giorgi et al., 2010; Marcoval et al., 2007). It is the second most common cancer with cutaneous metastasis after melanoma, which is associated with a poor prognosis, a short period of response to treatment, and an increased risk of development of distal metastasis (Freedman and Fowble, 2000; Lookingbill et al., 1993; Perez et al., 1994). However, early diagnosis and treatment have been found to improve disease outcomes

(González-Martínez et al., 2021; Teyateeti and Ungtrakul, 2021). Although cutaneous metastasis mainly occurs at the late stages of malignancies, it can be the first manifestation of breast cancer (Johnson et al., 2021). Lentigo maligna, a subtype of melanoma in situ, is another cutaneous malignancy mainly treated nonsurgically owing to its poor margin, anatomical locations, and advanced age of patients (Greveling et al., 2017; Read et al., 2016). Systemic treatments and radiation therapy have been used for the treatment of these unresectable cancers with modest benefits, significant side effects, and a high recurrence rate (Bichakjian et al., 2011; Greveling et al., 2017; Lookingbill et al., 1993). In patients with metastatic breast cancer, the tumor often becomes resistant to chemotherapy, and the efficacy of immune checkpoint blockade (ICB) therapy in metastatic triple-negative breast cancer is low (Emens et al., 2019; Voorwerk et al., 2019). Accordingly, innovative strategies are urgently needed to make CWD immunologically hot and responsive to ICB therapy.

Topical imiquimod (IMQ) is used for treating CWD and melanoma (Adams et al., 2012; Bichakjian et al., 2011; Salazar et al., 2017; Swetter et al., 2015). As a toll-like receptor 7 agonist, IMQ activates the innate immune response in the skin but does not directly activate adaptive immune cells (Adams et al., 2012). It has been previously demonstrated that the induction of an epithelial-derived cytokine, TSLP, protects against skin and breast cancer development (Azin et al., 2022; Boieri et al., 2022a; Cunningham et al., 2017; Demehri et al., 2016, 2012). This protection is mediated by T cells responding directly to TSLP (Boieri et al., 2022a; Demehri et al., 2016, 2012). In addition, it has been demonstrated that calcipotriol, a low-calcemic vitamin D analog and a topical inducer of TSLP, is a highly effective

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immunotherapy for breast and skin cancer (Boieri et al., 2022a; Demehri et al., 2016). Therefore, we hypothesized that the addition of a T-cell activating agent, including calcipotriol and retinoic acid (Ganti et al., 2017), to innate immune cell—activating agents, including toll-like receptor agonists, leads to potent topical immunotherapy against cutaneous malignancies. In this study, we demonstrate the efficacy of calcipotriol plus IMQ and retinoic acid plus IMQ in inhibiting cutaneous breast cancer and melanoma growth. Furthermore, we show that topical calcipotriol plus IMQ immunotherapy enables the efficacy of ICB therapy for breast cancer cutaneous metastasis in mice models.

RESULTS

Calcipotriol plus IMQ suppresses breast tumor development

To examine the efficacy of topical calcipotriol plus IMQ for breast cancer immunotherapy, we implanted primary breast tumor cells from MMTV-PyMt^{tg} (PyMt^{tg}) mice subcutaneously into the bilateral inguinal region of wild-type (WT) mice. When breast tumors reached 5 mm in diameter, mice received three applications of topical calcipotriol plus IMQ combination every 3 days (Figure 1a). Calcipotriol induced TSLP expression, which was detectable in the mice sera (Figure 1b). Calcipotriol plus IMQ treatment led to significant inhibition of breast tumor growth compared with calcipotriol, IMQ monotherapy, and vehicle control treatment ($P < 0.0001$) (Figure 1c and d). Whereas calcipotriol treatment alone showed a trend toward reduced breast tumor growth, IMQ monotherapy did not have any impact on breast tumor growth after the three applications (Figure 1d). These findings demonstrate the synergistic function of calcipotriol and IMQ in suppressing breast tumor growth.

Calcipotriol plus IMQ induces T-cell immunity in the breast tumors

Topical calcipotriol plus IMQ treatment resulted in a significantly higher CD3$^+$ T-cell infiltration into PyMt^{tg} breast tumors in WT mice than vehicle treatment ($P < 0.01$) (Figure 2a and b). Furthermore, CD4$^+$ CD3$^+$ T and FOXP3$^-$ CD4$^+$ CD3$^+$ effector T cells were significantly induced after calcipotriol plus IMQ treatment compared with the effect observed after calcipotriol and IMQ monotherapy as well as vehicle treatment (Figure 2a, c, and d). On the other hand, FOXP3$^+$ CD4$^+$ CD3$^+$ regulatory T-cell infiltration into breast tumors did not differ between the treatment groups (Figure 2e). Interestingly, calcipotriol monotherapy induced more CD4$^+$ effector T cells than IMQ, which showed no T-cell—inducing effect over vehicle control (Figure 2c and d).

Topical calcipotriol plus IMQ immunotherapy creates immunologically hot breast tumors responsive to ICB therapy

Next, we investigated whether T-cell induction by topical calcipotriol plus IMQ treatment in immunologically cold breast tumors could lead to their responsiveness to ICB therapy. Topical calcipotriol plus IMQ treatment of PyMt^{tg} breast tumors in combination with an intraperitoneal injection of anti—PD-1 antibody to the WT mice three times every 3 days led to significantly reduced tumor volume and tumor weight compared with topical combination alone, ICB monotherapy, and control treatment (topical control cream

plus IgG antibody) (Figure 3a—d). Breast tumor growth suppression by calcipotriol plus IMQ and anti—PD-1 antibody combination treatment led to a marked induction of CD3$^+$ T, CD4$^+$ CD3$^+$ T, and FOXP3$^-$ CD4$^+$ CD3$^+$ effector T cells in breast tumors compared with suppression by ICB monotherapy and control treatment (Figure 4a—d). FOXP3$^+$ CD4$^+$ CD3$^+$ regulatory T cells were not significantly changed in test groups compared with those in the control (Figure 4e). Although to a lesser degree, topical calcipotriol plus IMQ treatment suppressed breast tumor growth by inducing anti-tumor T-cell immunity, whereas ICB therapy alone had no effect on breast tumor growth (Figure 3). These findings demonstrate the synergy between topical calcipotriol plus IMQ treatment and systemic ICB therapy to create potent immunotherapy against breast cancer.

Topical TSLP induction plus innate immune activation suppresses melanoma growth

To extend our findings from breast cancer to melanoma, we used topical calcipotriol plus IMQ and other combinations of a TSLP inducer (i.e., retinoic acid [Figure 5a and b]) to suppress B16-F10 melanoma growth in WT mice. In addition to a significant reduction in tumor volume after combination therapy and monotherapy compared with that after vehicle control ($P < 0.0001$), three applications of calcipotriol plus IMQ and retinoic acid plus IMQ combinations led to significant inhibition of B16-F10 subcutaneous melanoma growth compared with monotherapy with calcipotriol ($P < 0.01$), retinoic acid ($P < 0.05$), or IMQ ($P < 0.0001$), as demonstrated by macroscopic images and tumor volume over time (Figure 5c—e). Interestingly, monotherapy with calcipotriol and retinoic acid was also associated with reduced tumor volume in comparison with the vehicle control ($P < 0.0001$ and $P = 0.0048$, respectively) (Figure 5e). Topical calcipotriol plus IMQ and retinoic acid plus IMQ treatments led to a significantly higher CD3$^+$ T, CD4$^+$ CD3$^+$ T, and FOXP3$^-$ CD4$^+$ CD3$^+$ effector T-cell induction in melanoma tumors than vehicle treatment ($P < 0.0001$ and $P < 0.01$, respectively) (Figure 6a—d). In contrast, FOXP3$^+$ CD4$^+$ CD3$^+$ regulatory T-cell infiltration into melanoma tumors did not differ between the treatment groups (Figure 6e). Collectively, these findings highlight the efficacy of topical TSLP induction plus innate immune activation for cutaneous malignancy treatment (Figure 7).

DISCUSSION

Our findings demonstrate that topical calcipotriol plus IMQ is significantly more effective than calcipotriol and IMQ monotherapies in blocking breast cancer and melanoma growth. Calcipotriol does not have any significant cell-autonomous cytotoxic impact on cells (Bagot et al., 1994; Guttmann-Gruber et al., 2018). It has been previously demonstrated that TSLP induction by calcipotriol is required for its protective function against skin and breast cancer, which is mediated by CD4$^+$ T cells responding directly to TSLP (Azin et al., 2022; Boieri et al., 2022a, 2022b; Cunningham et al., 2017; Rosenberg et al., 2019). Topical application of calcipotriol suppresses skin and breast tumors in WT but not in *Tslpr*^{KO} mice (Boieri et al., 2022a; Cunningham et al., 2017; Demehri et al., 2016). In addition, it has been demonstrated that a combination of calcipotriol plus 5-fluorouracil (5-FU) is an effective immunotherapy for keratinocyte carcinomas
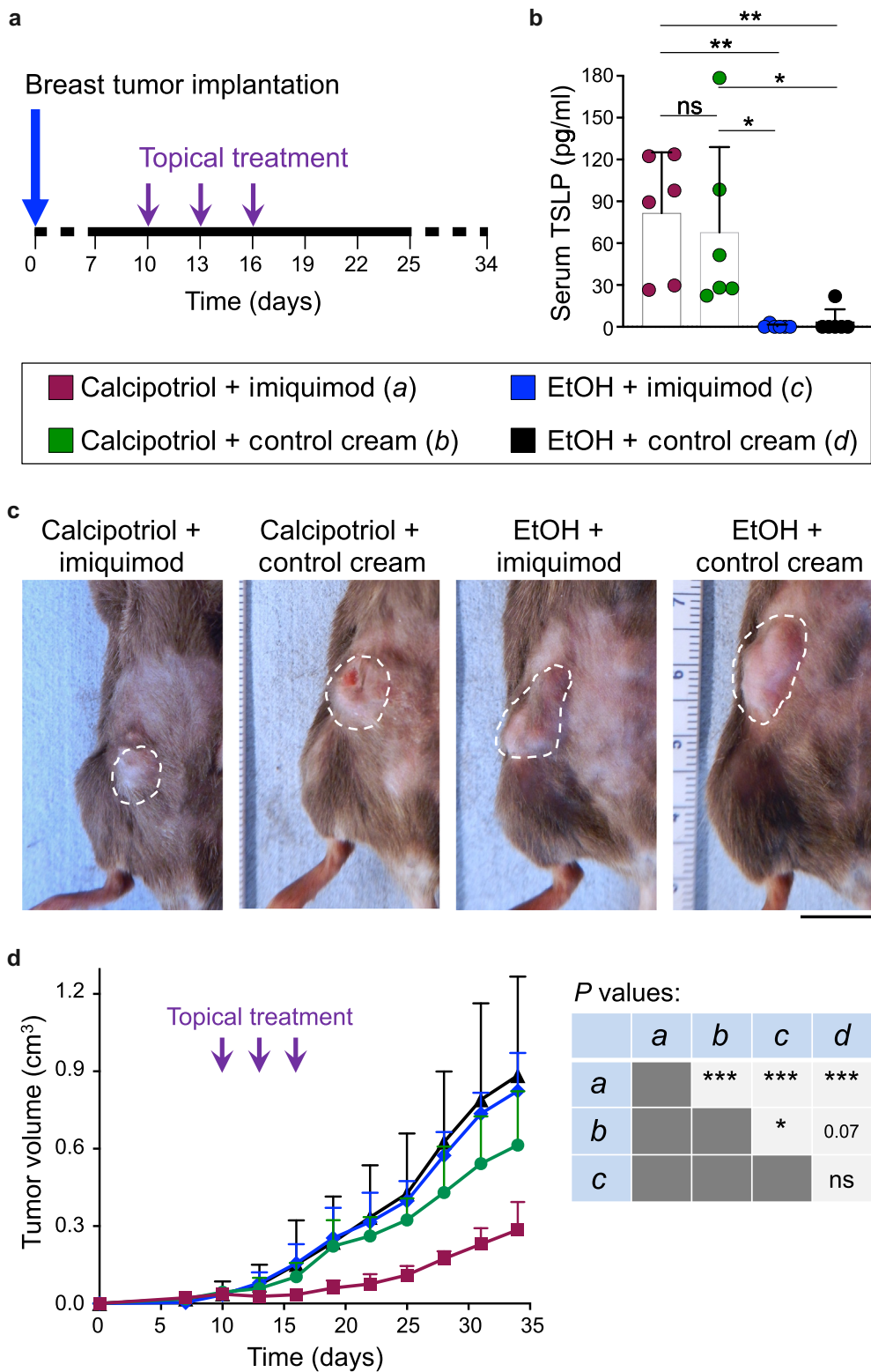
Figure 1. Calcipotriol plus imiquimod blocks breast tumor growth. (**a**) Schematic diagram of the experimental protocol used to determine the efficacy of calcipotriol plus imiquimod as a topical immunotherapy for breast cancer. PyMt[tg] primary breast cancer cells were implanted subcutaneously into the inguinal region of WT C57BL/6 mice. When tumors reached 5 mm in diameter (day 10), the following topical treatments were applied to the tumor sites three times every 3 days: (*a*) 80 nmol calcipotriol in 20 µl of 100% EtOH followed by 5% imiquimod cream, (*b*) 80 nmol calcipotriol in 20 µl of 100% EtOH followed by moisturizing (control) cream, (*c*) 20 µl 100% EtOH followed by 5% imiquimod cream, and (*d*) 20 µl 100% EtOH followed by moisturizing (control) cream. (**b**) Serum TSLP level of WT C57BL/6 mice 24 hours after the last topical treatments listed earlier (n = 6 per group, one-way ANOVA with Dunn's multiple comparisons test). (**c**) Representative macroscopic images of breast tumors in each treatment group at the endpoint (bar = 1 cm). (**d**) Tumor volume measurements over time in each treatment group. Purple arrows mark the treatment time points (n = 5 per group, two-way ANOVA with Sidak's multiple comparison test). Graphs show mean + SD; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$. Experimental data were verified in two independent experiments. EtOH, ethanol; ns, not significant; WT, wild-type.

leading to the elimination of actinic keratoses and the prevention of squamous cell carcinoma in a clinical trial with 131 participants (Azin et al., 2022; Cunningham et al., 2017; Rosenberg et al., 2019). The efficacy of calcipotriol plus 5-FU immunotherapy is associated with an increased number of CD4^{+}, CD8^{+}, and CD103^{+} tissue-resident memory T cells targeting the premalignant keratinocytes (Rosenberg et al., 2019). However, topical 5-FU is not an optimal treatment for nonkeratinocyte malignancies in the skin, likely because normal keratinocytes divide faster than the malignant cells, leading to off-target killing by 5-FU (Algarin et al., 2023; Singh et al., 2005). Thus, topical calcipotriol plus IMQ
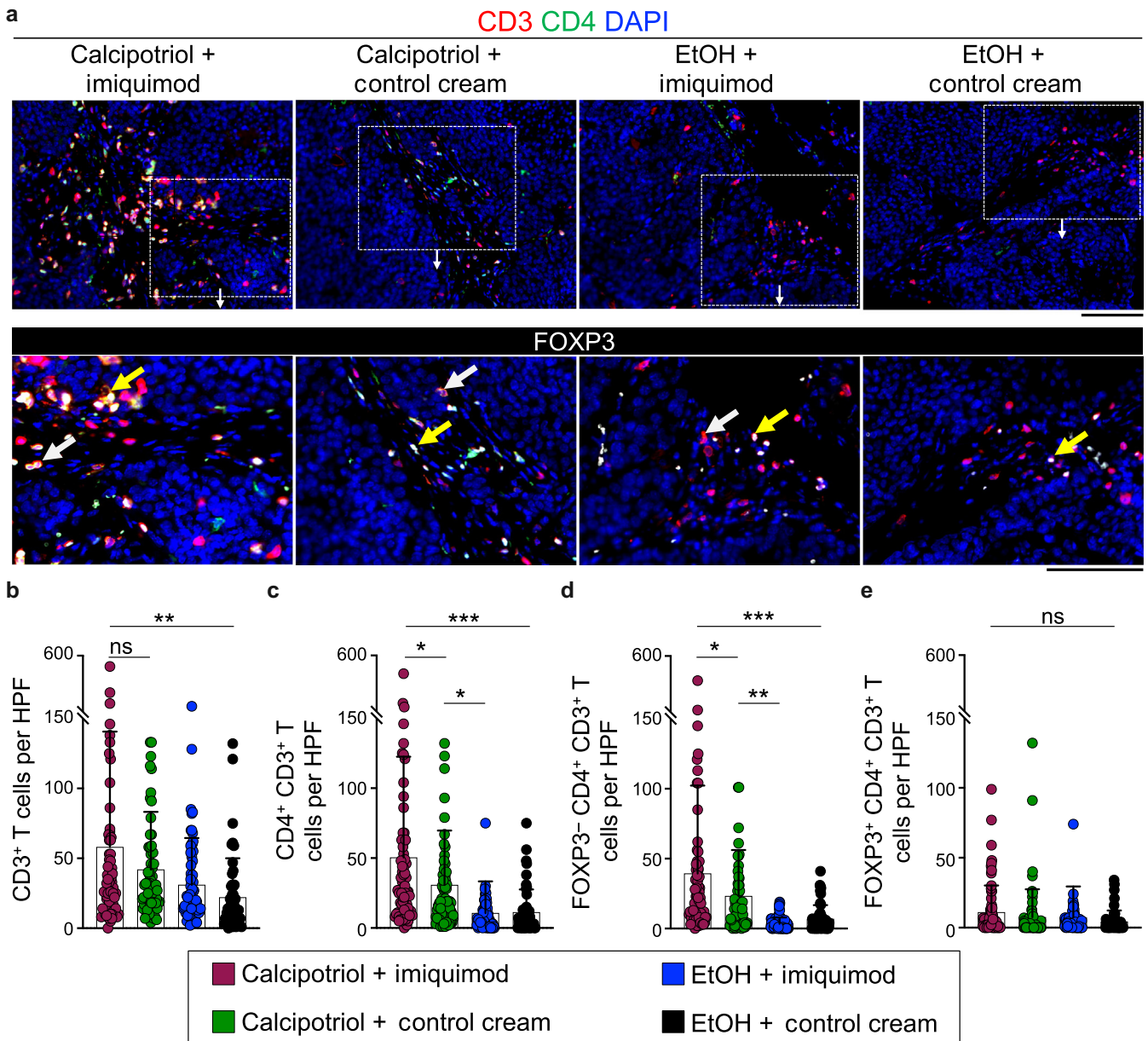
Figure 2. Calcipotriol plus imiquimod induces T-cell immunity in the breast tumor microenvironment. (**a**) Representative immunofluorescence images of CD3^{+}, CD4^{+} T cells, and FOXP3^{+} Tregs in calcipotriol plus imiquimod cream (n = 7), calcipotriol plus moisturizing (control) cream (n = 7), EtOH plus imiquimod cream (n = 8), and EtOH plus moisturizing (control) cream (n = 5) (upper row) and their magnified images (lower row). White arrows point to CD4^{+} CD3^{+} T cells, and yellow arrows point to FOXP3^{+} CD4^{+}CD3^{+} Tregs (bars = 100 μm). (**b**−**e**) Quantification of (**b**) CD3^{+} T cells, (**c**) CD4^{+} CD3^{+} T cells, (**d**) FOXP3^{−} CD4^{+} CD3^{+} effector T cells, and (**e**) FOXP3^{+}CD4^{+}CD3^{+} Tregs in the breast tumors in each treatment group. Cells were counted in 10 HPF images per tumor. Each dot represents cell counts from an HPF image. Experimental data were verified in two independent experiments. Graphs show mean + SD; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$; one-way ANOVA with Dunn's multiple comparisons test was performed. HPF, high power field; ns, not significant; Treg, regulatory T cell.

immunotherapy overcomes this limitation by directly activating immune cells in the tumor microenvironment.

Topical calcipotriol treatment has shown promising efficacy in the treatment of CWD (Bower et al., 1991). IMQ is also found to improve CWD response to systemic therapies (Demaria et al., 2013; Krishnasamy et al., 2018; Salazar et al., 2017). Topical IMQ induces proinflammatory cytokines in the tumor microenvironment of the CWD (Adams et al., 2012). Although IMQ can have a cytotoxic effect on skin cancer cell lines in vitro (Chuang et al., 2020; Huang et al., 2010), the direct cytotoxic impact of topical IMQ

on subcutaneous tumors may be limited. Consistently, we did not observe any appreciable efficacy for IMQ monotherapy in suppressing breast and melanoma tumor growth. By contrast, the innate immune response induced by IMQ complements the adaptive immune responses stimulated by radiation and systemic therapy (Apetoh et al., 2007; Demaria et al., 2013; Salazar et al., 2017). In a randomized clinical trial, patients with lentigo maligna who received 5% IMQ cream in combination with 0.1% tazarotene (topical retinoid) gel had a better prognosis than patients who received IMQ as a monotherapy (Higgins et al., 2015; Hyde
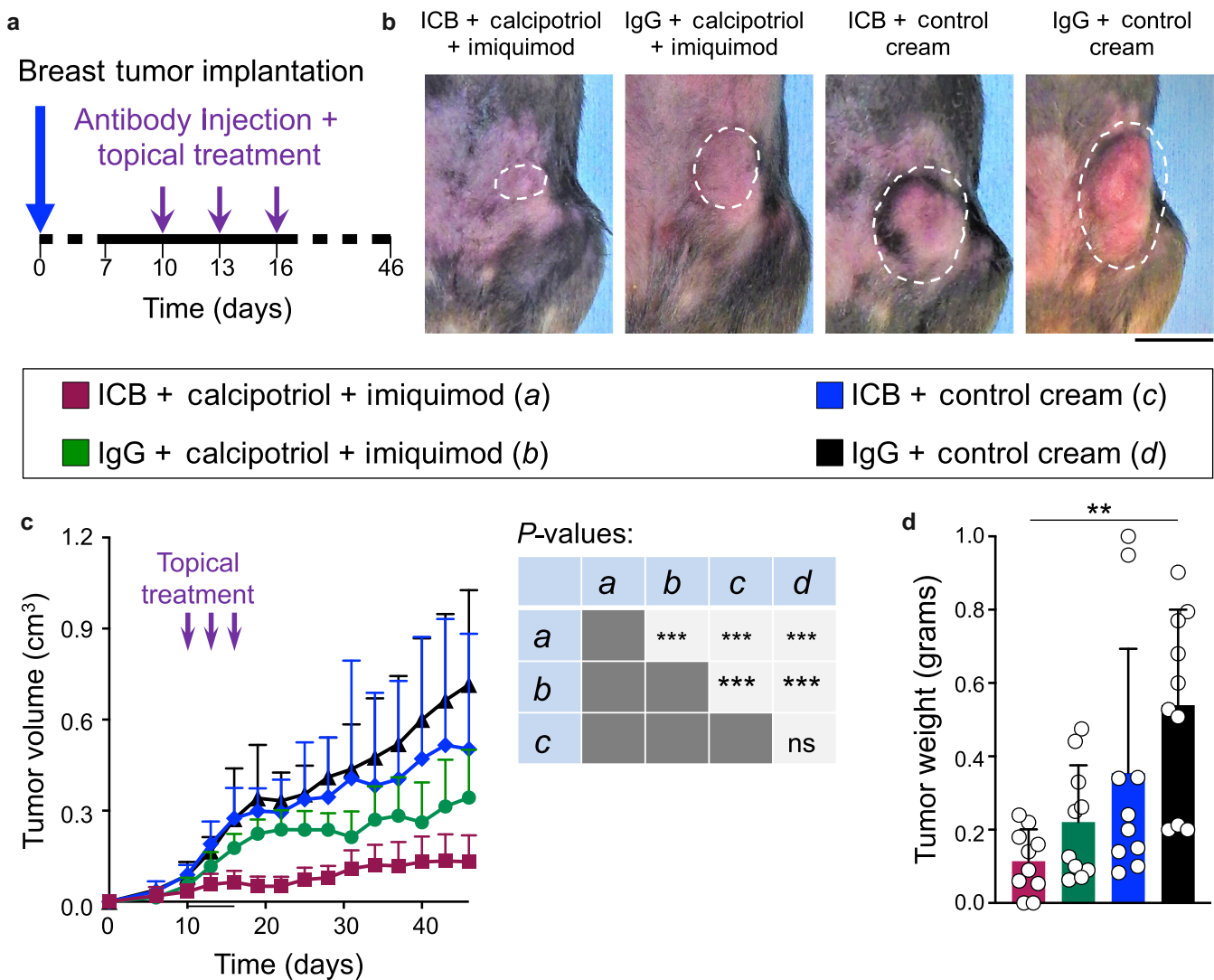
Figure 3. Topical calcipotriol and imiquimod immunotherapy synergize with ICB therapy to suppress breast tumor growth. (**a**) Schematic diagram of the experimental protocol used to determine the efficacy of calcipotriol plus imiquimod and ICB as a combination immunotherapy for breast cancer. PyMt[tg] primary breast cancer cells were implanted subcutaneously into the inguinal region of WT C57BL/6 mice. When tumors reached 5 mm in diameter (day 10), the following treatments were applied to the tumor sites three times every 3 days: (*a*) 80 nmol of calcipotriol in 20 µl of 100% EtOH followed by topical application of imiquimod cream and 250 µg anti−PD-1 antibody in 200 µl PBS IP injection (n = 6), (*b*) 80 nmol of calcipotriol in 20 µl of 100% EtOH followed by topical application of imiquimod cream and 250 µg IgG isotype in 200 µL PBS IP injection (*n = 7*), (*c*) 20 µL 100% EtOH followed by moisturizing cream and 250 µg anti-PD1 Ab in 200 µl PBS IP injection (n = 9), and (*d*) 20 µl 100% EtOH followed by control cream and 250 µg IgG isotype in 200 µl PBS IP injection (n = 9). (**b**) Representative macroscopic images of breast tumors in each treatment group at the endpoint (bar = 1 cm). (**c**) Tumor volume measurements over time in each treatment group. Purple arrows mark the treatment time points (n = 10 per group; two-way ANOVA with Sidak's multiple comparison test). (**d**) The endpoint tumor weights in each treatment group (n = 10 per group; one-way ANOVA with Dunn's multiple comparisons test). EtOH, ethanol; ICB, immune checkpoint blockade; IP, intraperitoneal; ns, not significant; WT, wild-type.

et al., 2012). In contrast, previous studies have shown that IMQ; retinoic acid derivatives; and topical chemotherapeutic agents, including 5-FU, are insufficient in treating melanoma in situ as monotherapies (Quigley and Halpern, 2013). Our findings demonstrate the role of adaptive immune system activation in augmenting the potency of IMQ for treating nonkeratinocyte cutaneous malignancies. This efficacy is mostly mediated by $CD4^+$ effector T cells (Boieri et al., 2022a). Considering the low efficacy and toxicity associated with radiation and systemic therapy (Tsoutsou et al., 2009), topical calcipotriol plus IMQ immunotherapy provides an innovative strategy to treat non-resectable skin cancers with minima6l side effects.

Our findings demonstrate that topical calcipotriol plus IMQ immunotherapy unleashes the efficacy of ICB therapy for the treatment of breast tumors in mouse models. Although ICB therapy has shown efficacy in treating a small subset of breast cancer with high PD-L1 expression (Cortes et al., 2020; Soliman et al., 2014; Voduc et al., 2010), most breast cancers, including CWD, are immune deserts and do not respond to ICB therapy (Emens et al., 2019; Voorwerk et al., 2019). Antigen-presenting cells, including dendritic cells and macrophages, in the tumor microenvironment are known to express PD-L1, which can predict response to ICB therapy (Herbst et al., 2014; Tumeh et al., 2014). Toll-like receptor agonists, including IMQ, increase the expression
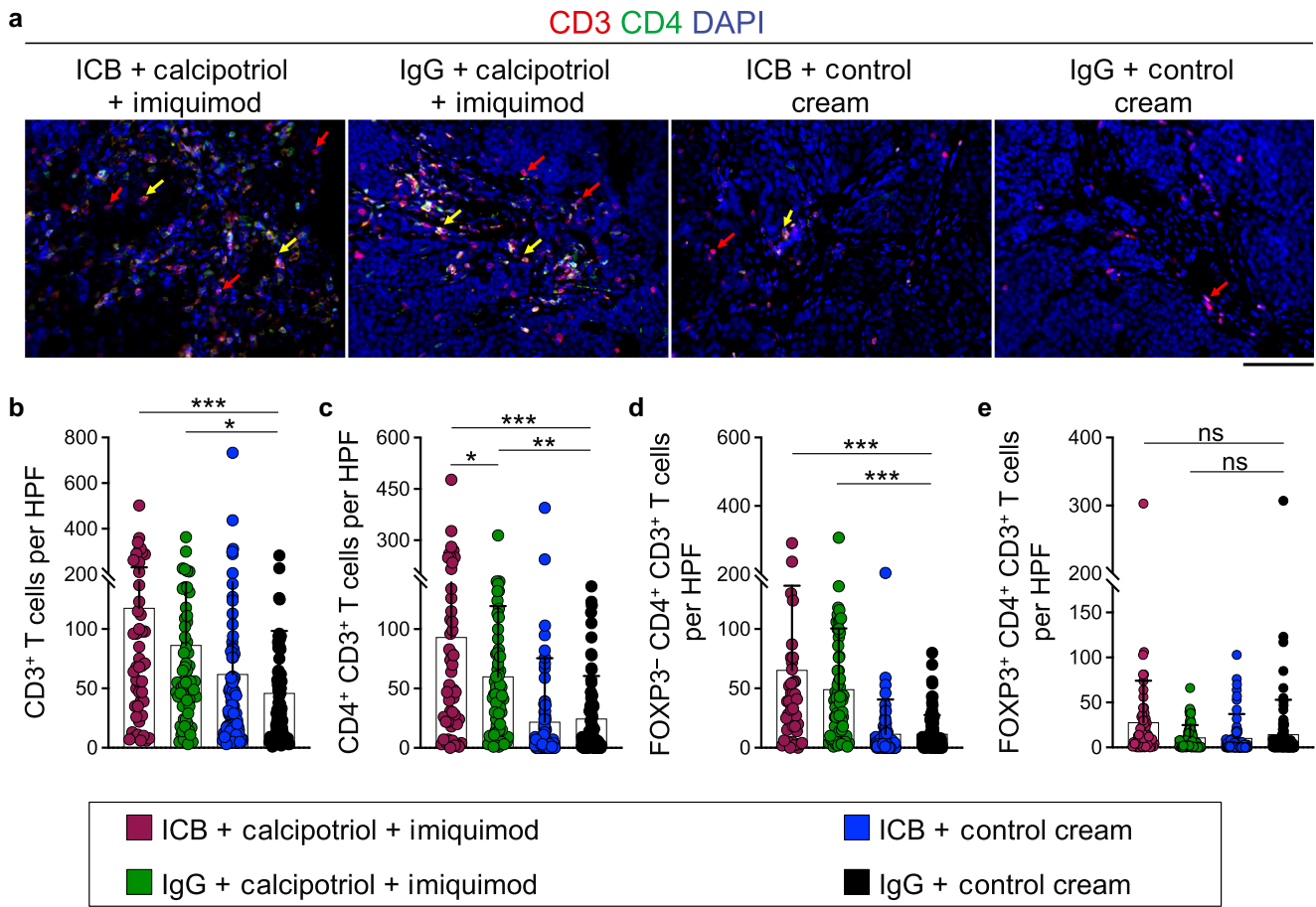
Figure 4. The combination of ICB therapy with topical calcipotriol plus imiquimod increases the effector T cells in breast tumors. (a) Representative immunofluorescence images of CD4$^+$ CD3$^+$ T cells in ICB plus calcipotriol plus imiquimod cream (n = 6), IgG plus calcipotriol plus imiquimod cream (n = 7), ICB plus control cream (n = 9), and EtOH plus control cream (n = 9). Red arrows point to CD3$^+$ T cells, and yellow arrows point to CD4$^+$CD3$^+$ T cells (bars = 100 μm). (b−e) Quantification of (b) CD3$^+$ T cells, (c) CD4$^+$ CD3$^+$ T cells, (d) FOXP3$^-$ CD4$^+$ CD3$^+$ effector T cells, and (e) FOXP3$^+$CD4$^+$CD3$^+$ Tregs in the breast tumors in each treatment group. Cells were counted in 9−12 HPF images per tumor. Each dot represents cell counts from an HPF image (one-way ANOVA with Dunn's multiple comparisons test). Graphs show mean + SD; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$. EtOH, ethanol; HPF, high power field; ICB, immune checkpoint blockade; ns, not significant; Treg, regulatory T cell.

of PD-L1 on dendritic cells (Lucas et al., 2020; Nishii et al., 2018; Tanaka et al., 2022; Wölfle et al., 2011). Thus, the combination of ICB with topical calcipotriol plus IMQ may lead to a synergistic effect to induce CD4$^+$ T-cell immunity as these T cells directly interact with antigen-presenting cells in the tumor microenvironment. Furthermore, topical calcipotriol plus IMQ immunotherapy transforms immunologically cold breast tumors into hot, immunogenic tumors by combining the innate signaling in the myeloid cells with direct CD4$^+$ T-cell stimulation by TSLP to activate robust adaptive immunity in the tumor. Therefore, topical calcipotriol plus IMQ immunotherapy creates an inflamed tumor microenvironment in which ICB therapy can operate to maximize antitumor immunity.

Simultaneous boost in antigen presentation by dendritic cells and macrophages and direct CD4$^+$ T-cell activation can explain the robust antitumor adaptive immunity induced by topical calcipotriol plus IMQ immunotherapy (Boieri et al., 2022a; Demehri et al., 2012). The capability to deliver these agents in a topical formulation creates a unique opportunity to effectively treat cutaneous malignancies such as melanoma and cutaneous metastasis from breast and other

internal cancers. Extramammary Paget disease (EMPD) is another attractive target for topical calcipotriol plus IMQ immunotherapy. EMPD is a rare adenocarcinoma of apocrine gland−bearing skin with a developmental origin similar to that of breast gland/cancer and is notoriously difficult to manage (Guarner et al., 1989; Heymann, 1993). Surgery for EMPD carries a high recurrence rate of 30% and causes significant morbidity owing to the location of the lesions, which mostly affect the groin and genitals (Hendi et al., 2004; Kanitakis, 2007; Tebes et al., 2002). In addition, IMQ has shown modest efficacy in treating EMPD (Machida et al., 2015; Molina et al., 2019). Therefore, topical calcipotriol plus IMQ immunotherapy may represent a breakthrough treatment for EMPD.

In conclusion, topical calcipotriol plus IMQ immunotherapy represents an effective class of therapeutic agents for treating unresectable cutaneous cancers, which may revolutionize the care of patients with these debilitating diseases. Further studies are needed to elucidate the precise immune mediators and underlying mechanisms of this combination immunotherapy. Clinical trials are warranted to investigate the efficacy of topical calcipotriol plus IMQ immunotherapy
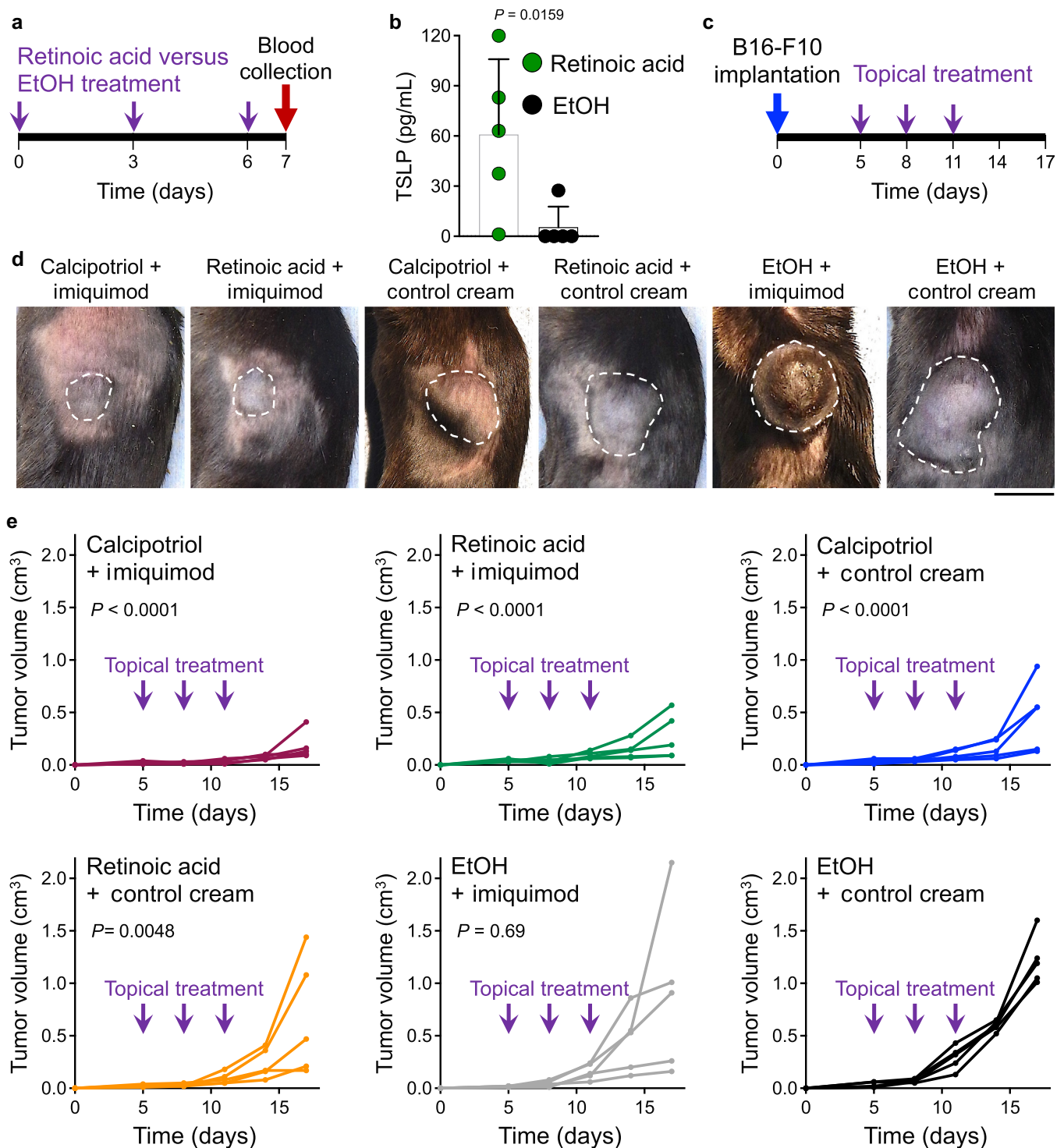
Figure 5. Calcipotriol plus imiquimod and retinoic acid plus imiquimod block melanoma growth. (**a**) Schematic diagram of the experimental protocol used to determine TSLP induction after topical retinoic acid treatment in WT C57BL/6 mice. A total of 20 nmol retinoic acid in 20 µl of 100% EtOH (test) versus 20 µl of 100% EtOH (carrier control) was applied on the back skin of mice three times at 3 days apart. Blood was collected 24 hours after the last topical treatment. (**b**) Serum TSLP levels in WT mice after treatment with topical retinoic acid versus EtOH control (n = 5 per group; Mann−Whitney *U* test). (**c**) Schematic diagram of the experimental protocol used to determine the efficacy of calcipotriol plus imiquimod and retinoic acid plus imiquimod immunotherapy for melanoma. B16-F10 melanoma cells were implanted subcutaneously into the flanks of WT C57BL/6 mice. When tumors become palpable (day 5), the following treatments were applied three times at 3 days apart: 20 nmol calcipotriol in 20 µl of 100% EtOH followed by 5% imiquimod cream, 20 nmol retinoic acid in 20 µl of 100% EtOH followed by 5% imiquimod cream, 20 nmol calcipotriol in 20 µl 100% EtOH followed by moisturizing (control) cream, 20 nmol retinoic acid in 20 µl 100% EtOH followed by control cream, 20 µl 100% EtOH followed by 5% imiquimod cream, and 20 µl 100% EtOH followed by control cream. (**d**) Representative macroscopic images of melanoma tumors in each treatment group at the endpoint (bar = 1 cm). (**e**) Tumor volume measurements over time in each treatment group. Purple arrows mark the treatment time points. n = 5 per group; two-way ANOVA with Sidak's multiple comparison test was performed. P-values are in comparison with EtOH plus control cream group. EtOH, ethanol; WT, wild-type.
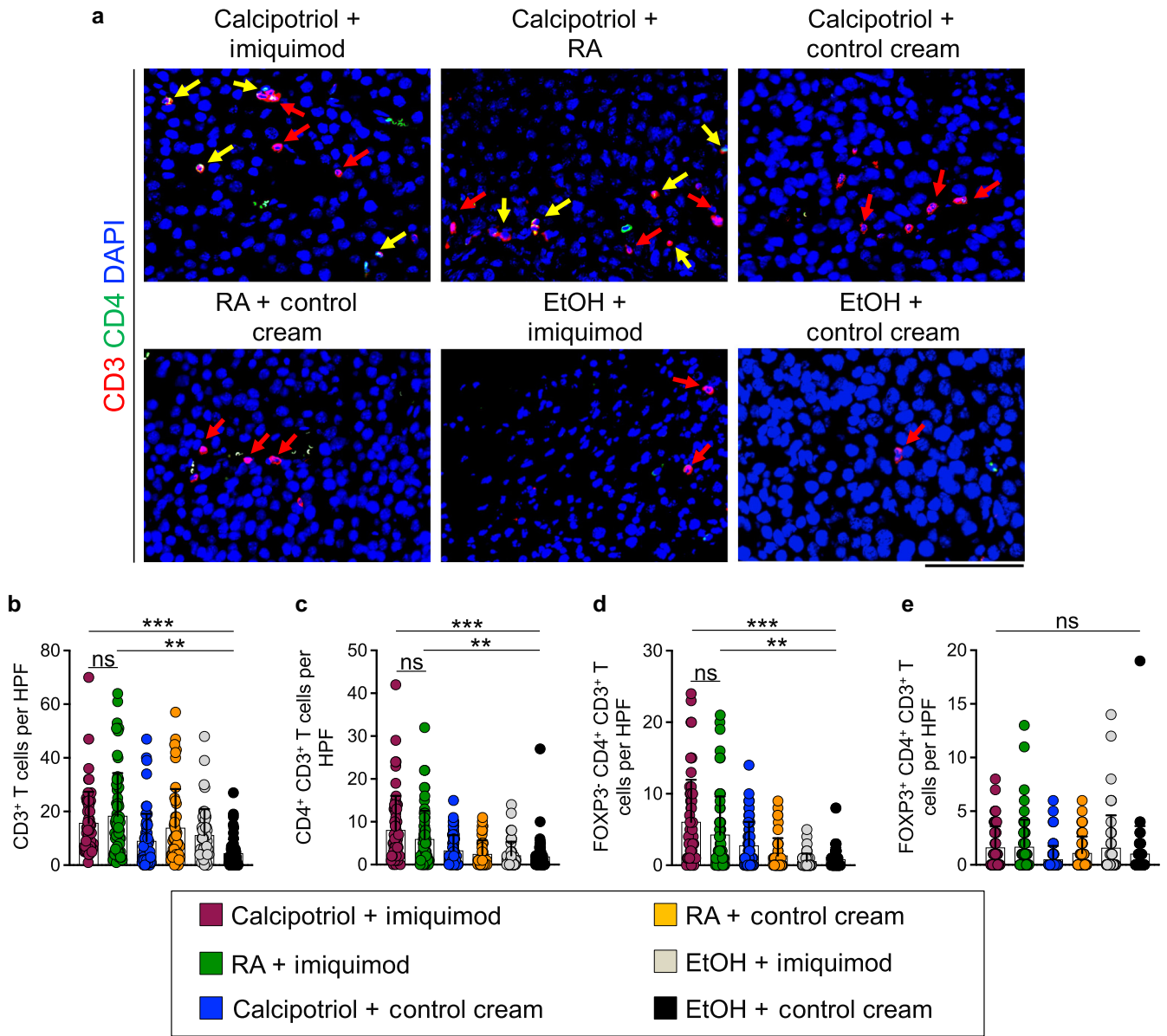
Figure 6. Calcipotriol plus imiquimod and retinoic acid plus imiquimod induce T-cell immunity in the melanoma microenvironment. (**a**) Representative immunofluorescence images of CD4+ CD3+ T cells in the following treatment groups: calcipotriol plus imiquimod cream (n = 6), retinoic acid plus imiquimod cream (n = 6), calcipotriol plus moisturizing (control) cream (n = 6), retinoic acid plus control cream (n = 5), EtOH plus imiquimod cream (n = 5), and EtOH plus control cream (n = 6). Red arrows point to CD3+ T cells, and yellow arrows point to CD4+CD3+ T cells. (**b**–**e**) Quantification of (**b**) CD3+ T cells and (**c**) CD4+ CD3+ T cells, (**d**) FOXP3− CD4+ CD3+ effector T cells, and (**e**) FOXP3+CD4+CD3+ Tregs in CD3/CD4/Foxp3-stained melanoma sections in each treatment group. Each dot represents cell counts from an HPF image (one-way ANOVA with Dunn's multiple comparisons test, bar = 200 μm). Graphs show mean + SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$. HPF, high power field; ns, not significant; Treg, regulatory T cell.

in various cutaneous malignancies, including EMPD, which are poor candidates for surgery.

In summary, topical TSLP induction in combination with innate immune cell–activating agents yields a synergistic efficacy for immunotherapy of nonkeratinocyte cancers in the skin.

MATERIALS AND METHODS

Mice

All mice were given food and water ad libitum and housed under specific pathogen-free conditions with a 12-hour light–dark cycle in an animal facility consistent with animal care regulations. C57BL/6 WT (C57BL/6NCr) mice were purchased from Charles River

Laboratory (strain code: 566, Wilmington, MA). MMTV-PyMt[tg] mice (PyMt[tg], a gift of David DeNardo, Washington University in St. Louis, St. Louis, MO) were bred in our facility to obtain mice to use in our studies. PyMt[tg] mice and WT mice were maintained on the C57BL/6 background. Subcutaneous breast cancer and melanoma cell injection were used to model CWD and cutaneous melanoma, respectively (Swenson et al., 2018; Vargo-Gogola and Rosen, 2007; Wojtynek et al., 2020). Age-matched female mice were used as tumor recipients in all experiments.

Breast cancer study

Breast cancer cells were obtained from PyMt[tg] mice in C57BL/6 background that develops spontaneous breast cancer resembling the
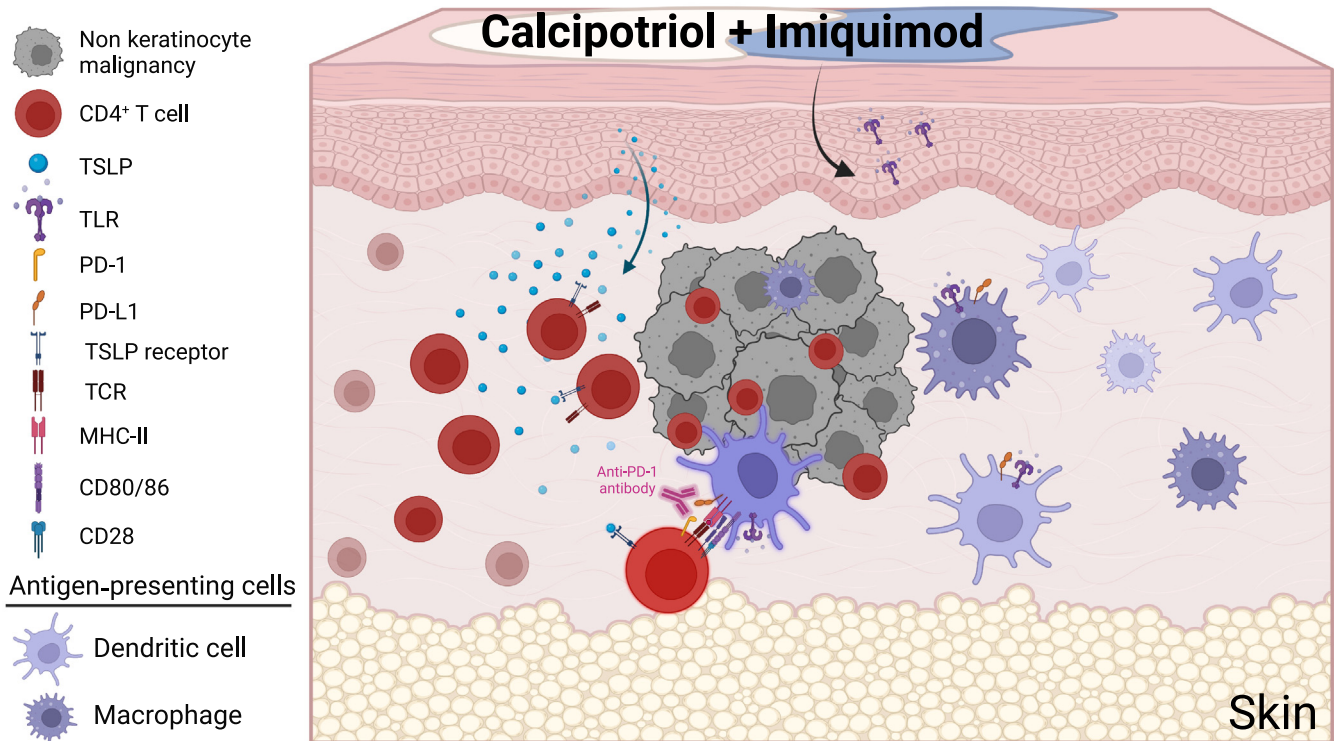
Figure 7. Schematic diagram demonstrating the synergistic effect of ICB with topical calcipotriol plus imiquimod in activating adaptive immunity against malignancies affecting the skin. The combination of ICB with topical calcipotriol plus imiquimod leads to a synergistic effect to induce CD4+ T-cell immunity because these T cells directly interact with antigen-presenting cells in the tumor microenvironment. ICB, immune checkpoint blockade.

luminal type of breast cancer in humans (Boieri et al., 2022a; Vargo-Gogola and Rosen, 2007). Breast tumors of PyMt[tg] mice were harvested before the tumors reached 2 cm in diameter. Tumors were excised and dissociated into a single-cell suspension. Breast cancer cells were resuspended at a concentration of 1×10^6 cells per 100 μl of 1:1 ratio of RPMI and Matrigel Membrane Matrix (Corning, Corning, NY). Inguinal regions of recipient WT C57BL/6 mice were shaved before cancer cell injection. A total of 1×10^6 cells were injected subcutaneously into the inguinal region at day 0. Topical treatments were applied when tumor sizes reached 5 mm in diameter. For the topical treatment experiments, mice were divided into four groups and were treated with either calcipotriol (80 nmol, Sigma-Aldrich, St. Louis, MO) or 100% ethanol (EtOH) as carriers directly on the tumor sites. After either calcipotriol or EtOH administration, tumor sites were treated with topical application of either 5% IMQ cream (Sigma-Aldrich) or moisturizing cream (control cream). Calcipotriol/EtOH and IMQ/control cream treatments were reapplied two more times every 3 days (Figure 1a). For ICB plus topical treatment, calcipotriol and IMQ or EtOH and moisturizing cream were applied to the tumor sites, followed by 250 μg anti−PD-1 antibody in 200 μl PBS or 250 μg IgG isotype in 200 μl PBS intraperitoneal injection (Figure 3a). Mice were monitored daily, and tumors were measured over time to determine the impact of topical treatments on breast cancer growth. At harvest, tumors were weighed and fixed in 4% paraformaldehyde (Sigma-Aldrich) for histological analysis.

Melanoma studies

B16-F10 melanoma cell line was used (ATCC, Manassas, VA). Melanoma cells were injected subcutaneously into the flanks of mice at 2×10^5 cells per 100 μl of 1:1 ratio of DMEM (Corning) at

day 0. Flank regions of recipient mice were shaved prior to cancer cell injection. Topical treatments were applied when tumors became palpable (day 5). Mice were divided into five groups and were treated with either calcipotriol (20 nmol, Sigma-Aldrich), retinoic acid (20 nmol, Sigma-Aldrich), or EtOH as carriers directly on the tumor sites. After either calcipotriol, retinoic acid, or EtOH treatment, tumor sites were treated with topical application of either 5% IMQ cream or moisturizing cream (control cream). Calcipotriol/retinoic acid/EtOH and IMQ/control cream treatments were reapplied two additional times at 3 days apart (Figure 5c). Mice were monitored daily, and tumors were measured over time to determine the impact of topical treatments on melanoma growth. At harvest, tumors were photographed and processed to make paraformaldehyde blocks and immunofluorescence staining.

Retinoic acid treatment

To investigate the effect of retinoic acid on TSLP level, five WT C57BL/6 mice received topical application of 20 nmol retinoic acid (Sigma-Aldrich) dissolved in DMSO (Corning) three times every 3 days. Mice were bled on the first day of treatment (day 0) and 1 day after the last treatment (day 7) (Figure 5a). TSLP levels in mice sera were measured using ELISA.

Tumor harvesting and blood collection

Mice were anesthetized using an isoflurane machine. After three minutes had elapsed, peripheral blood was collected by retroorbital bleeding using heparinized capillaries. The serum was isolated by centrifugation of the blood at 2,000g for 15 minutes and stored at −80 °C for future analysis. Breast and melanoma tumors were harvested and fixed in 4% paraformaldehyde and maintained at 4 °C overnight. Tissues were washed with PBS and dehydrated in EtOH.

The samples were processed and embedded in paraffin. A total of 5 µm sections of paraffin-embedded tissues were cut and stained for immunofluorescence staining.

Immunofluorescence

Tumor slides were rehydrated and permeabilized in 0.2% Triton X for 5 minutes. The slides were then heated in antigen unmasking solution (catalog number H-3300, Vector Laboratories, Burlingame, CA) at high pressure in a Cuisinart pressure cooker for 20 minutes. Slides were washed with 0.1% Tween 20 (catalog number P1379, Sigma-Aldrich) three times for 5 minutes each in 1x Dubellco's PBS. Then, the sections were blocked in blocking buffer containing 1% BSA (Thermo Fisher Scientific, Waltham, MA) and 5% goat serum (MilliporeSigma, Burlington, MA) for 1 hour and stained with anti-mouse primary antibodies, including anti-CD3 antibody (catalog number ab11089, Abcam, Cambridge, United Kingdom) and anti-CD4 antibody (catalog number ab183685, Abcam) overnight at 4 °C. After 12 hours, the sections were washed with 0.1% Tween 20, blocked in blocking buffer for the second time for 1 hour, and stained with anti-FoxP3 primary antibody (catalog number 12653, Cell Signaling Technology, Danvers, MA) overnight at 4 °C. On the following day, the sections were stained with fluorochrome-conjugated secondary antibodies and DAPI nuclear stain (Thermo Fisher Scientific). Slides were then mounted using 2−3 drops of mounting media (ProLong Gold Antifade reagent, Invitrogen, Waltham, MA). The Axio Scanner (Axio Scan.Z1, Zeiss, Jena, Germany) was used to scan the slides, and high-resolution images were obtained by a Zeiss Axio Observer Z1 (Zeiss, Oberkochen, Germany) and analyzed using the Zeiss ZEN Image Processing software. The cell population was quantified within ×20 magnified high-power fields by the HALO Image Analysis Platform (Indica Labs, Albuquerque, NM).

ICB therapy

Mice received intraperitoneal injection of 250 µg (∼10 mg/kg) anti-mouse PD-1 (clone 29F.1A12, catalog number BE2073, BioXCell, Lebanon, NH) or 250 µg mouse IgG isotypes antibody (catalog number 0107-01, Southern Biotech, Birmingham, AL) as control every 3 days.

Cell lines

B16-F10 WT cell lines were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and cultured at 37 °C/5% calcium dioxide.

ELISA

TSLP level was measured in mice serum using the LEGEND MAX Mouse TSLP ELISA Kit (BioLegend, San Diego, CA) following manufacturer instructions. Optical densities were measured on a Synergy Neo2 (Biotek, Winooski, VT) at 450 nm, and cytokine concentrations were calculated with a five-parameter logistic curve using Gen5 Microplate Reader and Imager Software (Biotek).

Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Bar graphs show mean + SD. Two-way ANOVA with Sidak's multiple comparison test was used to compare tumor growth over time between different groups. One-way ANOVA with Dunn's multiple comparisons test was used for serum TSLP level and the immune cell count comparisons. A $P < 0.05$ was considered significant. All error bars represent SD.

ETHICS APPROVAL

Animal studies were approved by Massachusetts General Hospital Institutional Animal Care and Use Committee. All mice were given food and water ad libitum and housed under specific pathogen-free conditions with a 12-hour light−dark cycle in the Massachusetts General Hospital animal facility consistent with animal care regulations.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request. No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST
SD is an inventor on a filed patent for the use of calcipotriol plus imiquimod for the treatment of cutaneous malignancies. The remaining authors state no conflict of interest.

AUTHOR CONTRIBUTIONS
Conceptualization: SD; Data Curation: MA, KHN, JH; Formal Analysis: MA, KHN, SD; Funding Acquisition: SD; Investigation: MA, KHN, JH, SD; Methodology: MA, KHN, JH, SD; Project Administration: SD; Resources: SD; Supervision: SD; Validation: MA, SD; Writing - Original Draft Preparation: MA, SD; Writing - Review and Editing: MA, SD

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